



Heterozygosity and fitness in a threatened songbird: blood parasite infection is explained by single-locus but not genome-wide effects

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Abstract

In non-pedigreed populations, insights into effects of inbreeding can be obtained by correlations between individual heterozygosity and fitness-related traits (HFCs). Using an information-theoretic approach, we explored whether heterozygosity of microsatellite markers, measured as internal relatedness (IR), is associated with infection by blood parasites (*Plasmodium*, *Trypanosoma*, or *Leucocytozoon*) in the threatened Aquatic Warbler (*Acrocephalus paludicola*). We also explored whether any of the markers is more influential than others, or than IR, in explaining blood parasitism (single-locus effects). While we observed that IR was a relatively important predictor of *Plasmodium* parasitism, we did not find strong evidence for IR to correlate with infection by the identified blood parasites, accounting for sex and population effects. Therefore, our data did not support negative inbreeding effects on blood parasite infection in the Aquatic Warbler. However, we found single-locus effects, such that individuals heterozygous at AW-03 and Ase19 had lower probability of infection by blood parasites pooled together and by *Plasmodium*, respectively. This indicates that these two markers are in linkage disequilibrium with unknown fitness loci which are related to resisting or clearing blood parasites, and which confer a heterozygote advantage in the Aquatic Warbler. Our results add to the growing evidence that single-locus effects contribute more to HFCs than formerly recognized and have implications for Aquatic Warbler conservation.

Keywords Heterozygosity-fitness correlations · General effects · Local effects · *Plasmodium* · *Trypanosoma* · Aquatic Warbler

Zusammenfassung

Heterozygosität und Fitness bei einem bedrohten Singvogel: Blutparasitenbefall wird durch Einzel-Lokus, aber nicht durch Genom-weite Effekte erklärt

Für Populationen, für die keine Informationen zu Abstammungsverhältnissen vorliegen, können Einblicke über Inzuchteffekte durch Korrelationen zwischen individueller Heterozygosität und dem Auftreten Fitness-relevanter Merkmale („heterozygosity-fitness-correlations“; HFCs) gewonnen werden. Für den bedrohten Seggenrohrsänger (*Acrocephalus paludicola*) untersuchten wir, ob Heterozygosität in Mikrosatelliten Markern, gemessen als interne Verwandtschaft („internal relatedness“; IR), mit der Befall durch Blutparasiten (*Plasmodium*, *Trypanosoma* oder *Leucocytozoon*) korreliert, in dem wir einen Informationstheoretischen Ansatz wählten. Wir untersuchten ebenfalls, ob irgendeiner der Marker einen größeren Einfluss hat als andere Marker, oder als IR, um den Befall mit Blutparasiten zu erklären (Einzel-Lokus Effekt). Während wir beobachteten, dass IR ein relativ wichtiger Prädiktor für Parasitenbefall mit *Plasmodium* ist, fanden wir keinen

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starken Hinweis darauf, dass IR mit Infektion durch die identifizierten Blutparasiten korreliert, wobei wir Geschlecht und Populationseffekte berücksichtigten. Daher unterstützen unsere Daten nicht die Hypothese eines negativen Einflusses von Inzucht auf den Befall mit Blutparasiten im Seggenrohrsänger. Wir fanden jedoch Einzel-Lokus Effekte. Individuen, die am AW-03 Locus heterozygot waren, hatten eine geringere Wahrscheinlichkeit generell mit Blutparasiten befallen zu sein, während Heterozygotie am Locus Ase19 negativ mit Plasmodium-Befall korrelierte. Dies deutet daraufhin, dass diese beiden Marker im Kopplungsungleichgewicht mit unbekanntem Fitness-relevanten Locus stehen, die mit der Resistenz oder dem Beseitigen von Blutparasiten einhergehen und die einen Heterozygoten-Vorteil beim Seggenrohrsänger bewirken. Unsere Ergebnisse tragen bei zu der wachsenden Erkenntnis, dass Einzel-Lokus Effekte stärker als HCFs sein können und sie haben Auswirkungen für den Schutz des Seggenrohrsängers.

Introduction

Heterozygosity and fitness correlations (HFCs) relate individual genetic variation to traits associated with fitness, giving insight into inbreeding depression and population viability (Reed and Frankham 2003; Grueber et al. 2008). HFCs have been found for survival, clutch size, breeding success, growth rates, and parasite infestation (Coltman et al. 1999; Rowe et al. 1999; Slate et al. 2000; Townsend et al. 2009; White et al. 2015; Cézilly et al. 2016). HFC research allows for a better understanding of evolutionary ecology of a species and can support its conservation (Grueber et al. 2008).

HFCs have been studied using molecular markers, such as microsatellites. They arise due to genome-wide effects (the general effect hypothesis) and/or single-locus effects (the local effect hypothesis) (Hansson and Westerberg 2002). General effects are observed when the heterozygosities of functional and marker (i.e., neutral) loci are associated (identity disequilibrium, ID), as they both respond in a similar way to inbreeding. Thus, if fitness is impaired by the lowered heterozygosity of genes, there is a correlation between fitness and marker heterozygosity. For example, in the Soay Sheep (*Ovis aries*), microsatellite heterozygosity was inversely correlated to parasitism by gastrointestinal nematodes and mortality caused by these parasites (Coltman et al. 1999). Local effects are found when the heterozygosity of a marker locus is related to that of a fitness-associated locus (linkage disequilibrium, LD), for instance due to physical linkage (Szulkin et al. 2010). The neutral marker hitchhikes with the selected locus and influences the heterozygosity metric more than do other markers under study. For example, heterozygosity at a single microsatellite locus predicted whether tuberculosis was localized or spread in the body of Wild Boars (*Sus scrofa*) (Amos and Acevedo-Whitehouse 2009).

HFCs have been used to study inbreeding especially in animal populations, which lack information on pedigrees to calculate the individual coefficient of inbreeding (F). F defines the probability that two alleles at a locus are identical by descent. In HFC studies on non-pedigreed populations, the underlying assumption is that F is inversely related to heterozygosity at a number of molecular markers

(multi-locus heterozygosity, MLH). This assumption has been subject to debate. On the one hand, the association between MLH and F was found to be weak when a low number of markers were used (Balloux et al. 2004; Slate et al. 2004). However, a more recent study (Forstmeier et al. 2012) made an important point that there is discrepancy between F calculated using a pedigree and the realized identity by descent, IBD (which determines the true rate of inbreeding), due to random events in Mendelian segregation. This discrepancy, rather than insufficient marker number, could explain the weak relationship between F and MLH. The strength of this relationship is also determined by variance in inbreeding, marker polymorphism, and the level of IBD (Hansson and Westerberg 2002; Csilléry et al. 2006; Ljungqvist et al. 2010; Taylor et al. 2010; Forstmeier et al. 2012). Overall, the literature shows that even at a modest number (e.g., up to 11), microsatellite markers can reliably detect HFCs that are attributable to inbreeding (Rowe et al. 1999; Slate et al. 2000; Hansson et al. 2004; Seddon et al. 2004; Forstmeier et al. 2012). To ensure legitimate inferences, it is important to test whether the markers of choice indicate inbreeding and whether there is variance in inbreeding, and to distinguish between the general and the local effects (Balloux et al. 2004; Pemberton 2004; David et al. 2007; Szulkin et al. 2010).

An important trait associated with fitness is resistance to parasites (Hamilton and Zuk 1982; Morand and Poulin 2000; Marzal 2012). In birds, some of the key parasites are vector-transmitted protozoans that invade the bloodstream. These include mainly the intra-erythrocytic hematozoans of the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, and the extracellular kinetoplastid of the genus *Trypanosoma* (Atkinson and Van Riper III 1991; Zídková et al. 2012). The fitness costs of blood parasitism are mediated by extensive damage in a variety of tissues, including destruction of erythrocytes, and activation of the immune system (Molyneux et al. 1983; Atkinson and Van Riper III 1991; Valkiūnas 2005). Infection with *Plasmodium*, the agent of avian malaria, negatively correlates with hatching success, brood provisioning rates, and fledging success (Knowles et al. 2010), survival (Stone et al. 1971; Beier et al. 1981; Krama

et al. 2015; Ilgūnas et al. 2016; Townsend et al. 2018b), predation risk (Møller and Nielsen 2007; Krama et al. 2015), song quality and output (Gilman et al. 2007), and fat score during migration (Shurulinkov et al. 2012). In passerines, experimental medication with anti-malarial drugs showed that hematozoan blood parasites negatively affect clutch size, hatching success, parental provisioning rates, nestling growth, fledging success, and survival (Merino et al. 2000; Marzal et al. 2005; Knowles et al. 2010; Martínez-de La Puente et al. 2010). Infections with trypanosomes have been associated with later arrival to breeding grounds in spring, siring fewer offspring, and less effective nest defence against a predator relative to uninfected individuals (Rätti et al. 1993; Hakkarainen et al. 1998; Dyrz et al. 2005).

As in birds hemoparasite infection is an important fitness determinant, it is crucial to identify its underlying variables. One of the traits with a potential effect on infection occurrence is the genetic diversity of the host (Amos et al. 2001; Frankham 2005). However, the link between genetic diversity and blood parasitism in birds is not well understood, because studies that addressed it have been scarce and yielded mixed results (MacDougall-Shackleton et al. 2005; Vallender et al. 2012; Boerner et al. 2013; Ferrer et al. 2014; Townsend et al. 2018a). In addition, only one study focused on a species with a small population (Ortego et al. 2007). In species with a small and fragmented population, studying HFCs is crucial, as it can benefit their conservation (Reed and Frankham 2003; Grueber et al. 2008).

Here, using an information-theoretic approach, we investigated whether the probability of infection with vector-transmitted blood parasites is predicted by (1) host MLH and/or, (2) single-locus effects of specific microsatellite loci in the Aquatic Warbler (*Acrocephalus paludicola*). This globally threatened passerine, a habitat specialist breeding in isolated wetlands, underwent a steep decline in population size (Briedis and Keišs 2016; Flade et al. 2018), which could have lowered its genetic diversity and increased inbreeding rates. The Aquatic Warbler is infected by blood parasites of the genera *Leucocytozoon*, *Plasmodium*, and *Trypanosoma*; in males, infection with trypanosomes negatively correlates with fitness-related traits (Dyrz et al. 2005; Neto et al. 2015; Kubacka et al. 2019). These findings, converged with the previous results on haemosporidian parasites in other passerines (Merino et al. 2000; Marzal et al. 2005; Knowles et al. 2010; Martínez-de La Puente et al. 2010), indicate that blood parasitism likely affects the fitness status of the Aquatic Warbler. Therefore, this passerine comes as a good model to study HFCs related to blood parasite infection in species of conservation concern.

Materials and methods

Study area and sampling

We sampled two core breeding populations of the Aquatic Warbler in Eastern Poland, differing by size and degree of fragmentation. The Biebrza Valley population (central position: 53°17'10.6" N, 22°33'41.9" E) is one of the largest (c. 2700 singing males), and occupies extensive and rather continuous mire habitat. The Polesie population (central position: 51°21'48.9" N, 23°17'21.4" E), located c. 250 km away, holds approximately 600 singing males and breeds at several small mires scattered across a large area.

In total, we obtained blood samples from 144 Aquatic Warblers between May and August 2014 (see Online Resource 1 for a list of samples). For the purpose of this analysis, five immature individuals were excluded, as their sex was unknown and because age affects blood parasite infection (Marzal et al. 2016). Hence, the sample sizes were 50 in the Biebrza Valley (17 females, 33 males) and 89 in Polesie (18 females, 71 males). The birds were sexed by the presence of cloacal protuberance (males) or brood-patch (females) and aged by plumage (Svensson 1992). We collected the blood by puncturing the brachial vein and stored the samples in 96% ethanol until laboratory analysis.

DNA extraction and microsatellite markers

We genotyped 17 microsatellite loci (Table 1), which—due to the project being carried out in two different laboratories—were analysed in two pools: (1) AW-02, AW-03, AW-07, AW-08, AW-10 and AW-11 (Salewski et al. 2012), and (2) AW-01, AW-04, AW-05, AW-06, AW-09 (Salewski et al. 2012), AW-12 (*Salewski unpubl.*, used with permission; see Online Resource 2 for primer sequences), Aar3 (Hansson et al. 2000), Fhu2 (Primmer and Ellegren 1998), Pocc8 (Bensch et al. 1997), Ase19, and Ase26 (Richardson et al. 2000). In pool 1, DNA was extracted using a phenol–chloroform protocol (Sambrook and Russell 2001) and the PCR was run in 20 µl volumes with 1× enhancer, 1× Taq incubation buffer without MgCl₂, 0.2 mM of dNTPs, 0.5 µM of each forward (5' labelled with the dyes Hex, Fam or Cy3) and reverse primer, 0.16 mM MgCl₂, 0.5 U Taq polymerase (Merck), and 10–100 ng DNA in. The PCR program started with an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min, and ended with a final extension step of 72 °C for 10 min. In pool 2, DNA was extracted using an ammonium acetate precipitation protocol based on Bruford et al. (1998) and the markers were amplified in multiplexes, using the Multiplex Manager v.1.2 (Holley and Geerts 2009) and a multiplex PCR kit (Qiagen)

Table 1 Microsatellite loci used to genotype Aquatic Warblers

	Locus	Proportion missing genotypes	Na	Ho	He	<i>p</i> (HWE)	Null allele freq. Oosterhout	
Biebrza	AW-01	0.000	1	0.000	0.000	n.a	0.000	
	AW-02	0.000	14	0.800	0.843	0.008	0.025	
	AW-03	0.000	6	0.260	0.254	0.998	−0.002	
	AW-04	0.020	2	0.571	0.465	0.108	−0.125	
	AW-05	0.040	2	0.021	0.021	0.942	−0.011	
	AW-06	0.020	5	0.878	0.579	0.001*	−0.517	
	AW-07	0.000	2	0.440	0.471	0.640	0.033	
	AW-08	0.000	6	0.560	0.618	0.952	0.054	
	AW-09	0.060	2	0.021	0.021	0.941	−0.011	
	AW-10	0.020	7	0.306	0.544	0.000*	0.199 ^a	
	AW-11	0.000	5	0.360	0.621	0.000*	0.198 ^a	
	AW-12	0.000	1	0.000	0.000	n.a	0.000	
	Fhu2	0.020	13	0.857	0.881	0.264	0.015	
	Pocc8	0.120	9	0.659	0.722	0.342	0.034	
	Ase19	0.020	3	0.510	0.488	0.000*	−0.037	
	Ase26	0.060	14	0.511	0.793	0.000*	0.168 ^a	
	Aar3	0.280	16	0.583	0.884	0.000*	0.169 ^a	
	Polesie	AW-01	0.011	2	0.011	0.011	0.957	−0.006
		AW-02	0.045	17	0.929	0.862	0.766	−0.042
AW-03		0.000	5	0.292	0.276	0.013	−0.015	
AW-04		0.034	3	0.477	0.464	0.977	−0.013	
AW-05		0.090	2	0.012	0.012	0.955	−0.006	
AW-06		0.000	5	0.775	0.553	0.000*	−0.322	
AW-07		0.000	4	0.461	0.497	0.918	0.035	
AW-08		0.000	6	0.753	0.716	0.223	−0.031	
AW-09		0.022	2	0.069	0.067	0.739	−0.035	
AW-10		0.000	8	0.449	0.692	0.000*	0.171 ^a	
AW-11		0.022	4	0.391	0.635	0.000*	0.186 ^a	
AW-12		0.034	2	0.093	0.089	0.651	−0.048	
Fhu2		0.067	15	0.904	0.873	0.927	−0.018	
Pocc8		0.090	11	0.704	0.788	0.002*	0.055	
Ase19		0.090	7	0.457	0.478	0.975	0.028	
Ase26		0.034	14	0.628	0.739	0.000*	0.074 ^a	
Aar3		0.326	17	0.633	0.896	0.000*	0.146 ^a	

All calculations are population-wise. Loci AW-01, AW-10, AW-11, AW-12, Ase26, and Aar3 were excluded from the analysis

Na number of alleles, Ho observed, He expected heterozygosity, *p* (HWE) *p* value of the Hardy–Weinberg test

*Significant after sequential Bonferroni correction

^aPresence of null alleles according to Microchecker

in 20 µl volumes, with 1 × Qiagen Multiplex PCR Master Mix, 0.2 µM of each forward (5' labelled with Hex, Fam or Cy3) and reverse primer, 1 × Q-Solution and 10–100 ng DNA, completed with RNase-free H₂O. The PCR products were sized with an ABI 3130 Avant sequencer (pool 1) and an ABI 3730xl DNA Analyzer (pool 2). Electropherograms were scored by one person (JK) using the PeakScanner software v. 1.0 (Applied Biosystems).

Both extraction methods that we applied produce yields of comparable DNA quality and quantity (Sameer et al. 2009; Mirmomeni et al. 2011). The pattern of null alleles in our sample (see Table 1) was similar to the one found in a sample collected from Aquatic Warblers in the Biebrza Valley and Polesie in another project and typed at the same microsatellite loci but with a different PCR protocol (Kubacka unpubl.). Thus, we had confidence that employing

two different PCR protocols for our samples was unlikely to create a bias in null allele pattern and hence allele frequencies.

We used Genealex 6.5 (Peakall and Smouse 2006, 2012) to check the markers for deviation from the Hardy–Weinberg rule and the Genepop web interface (Raymond et al. 1995; Rousset 2008) to test for linkage disequilibrium, applying the sequential Bonferroni correction to the obtained p values. We assessed null allele frequency using the Microchecker and the Oosterhout estimator, which is well suited for non-equilibrium populations (Van Oosterhout et al. 2006).

Heterozygosity and identity disequilibrium

As a heterozygosity metric, we used the internal relatedness (IR). IR is a measure of parental relatedness that weighs heterozygosity by allele frequency. It is calculated with the formula:

$$\frac{2H - \sum f_i}{2N - \sum f_i^2}$$

where H is the number of homozygous loci in an individual, N is the total number of loci, and f_i is the frequency of the i th allele in the genotype. IR assumes values between -1 (all heterozygous) and 1 (all homozygous). Negative IR values indicate more outbred and positive IR values suggest more inbred individuals within the studied sample (Amos et al. 2001). In the case of a species with fragmented population and low expected genetic diversity due to a steep recent decline, IR appears to be superior to homozygosity by loci (HL), which is better suited for open populations with relatively high immigration rates and heterozygosity (Aparicio et al. 2006). IR is a measure of heterozygosity that yields the strongest HFCs and is highly correlated with other measures, such as heterozygosity, standardized heterozygosity, and homozygosity by loci (Chapman et al. 2009; Forstmeier et al. 2012).

To test whether the marker loci showed ID, we tested for a heterozygosity–heterozygosity correlation (HHC) (Balloux et al. 2004). The set of marker loci was randomly divided in half to see whether the heterozygosity of the first group is correlated with that of the second group across individuals. This procedure was repeated 1000 times and the mean correlation coefficient was calculated with its confidence interval. If it is significantly greater than zero, a genome-wide effect is inferred that is likely due to inbreeding. In addition, we computed the g_2 statistic, which is an indicator of variance of inbreeding in the population. g_2 quantifies the covariance of heterozygosity at the studied loci standardized by their average heterozygosity (David et al. 2007).

We calculated IR, HHC, and g_2 in the R environment (v. 3.5.1; R Core Team 2018). IR and HHC were quantified

using the package Rhh (v. 1.0.2; Alho et al. 2010) and the g_2 statistic was calculated with the package inbreedR (v. 0.3.2; Stoffel et al. 2016), running 1000 permutations.

Identification and diversity of blood parasites

The blood samples were molecularly screened for the presence of blood parasites in a different project (Kubacka et al. 2019). In brief, the DNA samples extracted following the phenol–chloroform protocol were used to PCR-amplify a 478 bp fragment of the mitochondrial cyt b gene to detect *Leucocytozoon*, *Haemoproteus*, and *Plasmodium* (Hellgren et al. 2004), and a 326 bp fragment of the small subunit ribosomal RNA (SSU rRNA) gene to identify *Trypanosoma* (Sehgal et al. 2001). PCR products of positive samples were purified and sequenced. The obtained sequences were aligned to sequences available in the MalAvi database (Bensch et al. 2009) for *Leucocytozoon*, *Haemoproteus*, and *Plasmodium*, and in GenBank for *Trypanosoma* (BLASTN, best hit match) (Kubacka et al. 2019).

Statistical analysis

We performed the analysis in the R environment (v. 3.5.1; R Core Team 2018). We used the Akaike information criterion corrected for small-sample size (AICc) and the information-theoretic approach for inference (Burnham and Anderson 2002). In this approach, in contrast with the null hypothesis testing, the researcher creates an a priori set of biologically plausible candidate models, which are given relative AICc ranks corresponding to how well they explain the data. The best-ranking model (or a subset of best models) is then selected, with models within 2 AICc units relative to the top model (which has the lowest AICc) considered to be highly supported (Burnham and Anderson 2004). Each model is given a quantitative measure of relative support, by computing model likelihood (the relative likelihood of the model in the candidate set, given the data), the Akaike weight (ω AICc; the probability of the model for a given set of candidate models), and the evidence ratio (the ratio of the model's probability to the probability of the top model).

We ran two separate analyses, in both of which we used generalized linear models with binomial error structure and logit link, fitted using the 'glm' function from the stats package. First, we assessed the general heterozygosity effects on infection probability. We constructed three sets of candidate models, with the infection status by blood parasites pooled (candidate set 1), *Plasmodium* (candidate set 2), and *Trypanosoma* (candidate set 3) as a binary response variable, respectively. Because infection by *Leucocytozoon* was detected only in one individual (Kubacka et al. 2019), this genus was included in the pooled parasites models, but we did not run a separate analysis for

it. In each of the candidate sets, the global (full) model included IR, as well as region (Biebrza or Polesie), sex (male or female) and all the two-way interactions. This allowed us to control for factors that affect infection rates and/or HFCs, such as population structure, sex, or habitat (Reid et al. 2007; Brekke et al. 2010; Szulkin et al. 2010; Sehgal 2015; Arct et al. 2017; Kubacka et al. 2019). Each candidate set contained a null model, which assumed that infection probability was constant in all individuals. By choosing this all-subset approach, we were also able to quantify the importance of IR in explaining infection, relative to sex and population. The importance of a variable is calculated by summing the Akaike weights across all the models in the set that contain this variable (Burnham and Anderson 2002). Second, to test for single-locus effects, we again constructed three sets of candidate models, with the response variables as above and the explanatory variables being the heterozygosities of the microsatellite loci, coded as 0 (homozygous) or 1 (heterozygous). Since six loci were discarded from the analyses of single-locus effects (see “Results”), there were 11 locus models. Each of the three candidate sets also included a model assuming that infection probability varied only with IR and a null model, yielding a total of 13 models in each candidate set. This enabled us to see whether any single locus explained the given infection status better than did other loci or IR, and better than the null model. Missing values (see Table 1) for a given locus were replaced by its mean heterozygosity (Szulkin et al. 2010). To ensure that missing values did not bias the results, we re-ran all the models taking standardized heterozygosity (SH) instead of IR. SH is best applied to a data set in which not all individuals are genotyped at all the loci. It is calculated by standardizing individual heterozygosity at the genotyped loci by their mean heterozygosity, thus making heterozygosity independent of the loci that are genotyped (Coltman et al. 1999).

In both analyses, the sum contrasts were used, IR was centred, and estimates were model-averaged across the 95% confidence set of the best models (i.e., a set including the top models the cumulative ω AICc of which equals or just exceeds 0.95) to account for model selection uncertainty (Burnham and Anderson 2002). We applied the package AICcmodavg (v. 2.2–2) for model selection (Mazerolle 2019), the MuMIn package (v. 1.43.9) for calculation of model-averaged estimates along with their confidence intervals (Bartoń 2019), and the ResourceSelection package (v. 0.3–5) to run the Hosmer–Lemeshow goodness of fit test and assess whether the global model fitted the data (Hosmer and Lemeshow 2000; Lele et al. 2019).

Results

Four loci showed null alleles and two loci were monomorphic in the Biebrza population (Table 1), and we therefore discarded these six markers in further analyses. We did not detect LD among the markers (all sequential Bonferroni-corrected p values > 0.05). IR ranged from -0.559 to 0.797 (mean \pm SD -0.034 ± 0.211) and did not vary by sex ($F_{1,137} = 1.66$, $p = 0.200$) or population ($F_{1,137} = 0.88$, $p = 0.351$). The HHC was not different from zero for the overall data set (mean $r = -0.053$, 95% CI -0.138 to 0.050), Biebrza (mean $r = 0.067$, 95% CI -0.079 to 0.211), or Polesie (mean $r = -0.117$, 95% CI -0.248 to 0.015). Neither was g_2 different from zero, whether for all the individuals ($g_2 = -0.003$, 95% CI -0.019 to 0.015), Biebrza ($g_2 = 0.015$, 95% CI -0.019 to 0.059), or Polesie ($g_2 = -0.011$, 95% CI -0.026 to 0.006). The identified blood parasite lineages and infection prevalence were reported elsewhere (Kubacka et al. 2019). In brief, 31%, 12%, and $< 1\%$ individuals were infected by *Plasmodium*, *Trypanosoma*, and *Leucocytozoon*, respectively. No *Haemoproteus* infections were detected.

Heterozygosity and blood parasites: multi-locus effects

Probability of infection by blood parasites pooled was best explained by the constant infection model. The IR model, which was within the ≤ 2 Δ AICc subset, ranked third and had about twice lower probability relative to the null model (Table 2). The odds ratio (OR) corresponding to the model-averaged estimate of the IR effect (Table 3) suggested that probability of infection by any blood parasite increases about twofold with one unit increase in IR (Fig. 1a). However, the confidence interval of the IR estimate spanned zero, indicating that the effect of IR is not truly different from null. The relative importance of IR, sex, and population was 0.42, 0.46, and 0.36, respectively. The full model fitted the data well, as there was no significant difference between the fitted and the observed data ($X^2 = 3.98$, $df = 8$, $p = 0.859$). An analysis run on the data set excluding the individual parasitized by *Leucocytozoon* yielded similar results (not shown).

Infection by *Plasmodium* was also best explained by the constant infection model, with the IR model ranking second and having a similar probability (Table 2). The estimate of the IR effect suggested an approximately twofold increase of *Plasmodium* infection probability with one unit increase in IR (Fig. 1b); however, its confidence intervals also overlapped zero (Table 3). The relative importance of IR, sex, and population in explaining *Plasmodium* infection was 0.60, 0.61, and 0.40, respectively. The global model fitted the data well ($X^2 = 5.97$, $df = 8$, $p = 0.651$).

Table 2 Model selection results for the relationship between probability of infection by blood parasites and internal relatedness (IR), sex, and the population of origin

Model	K^a	AICc ^b	Δ AICc ^c	Evidence ratio	ω AICc ^d	Log-likelihood	Cumulative AICc
Blood parasites pooled							
Null	1	186.81	0.00	1.00	0.25	−92.39	0.25
Sex	2	187.97	1.15	0.56	0.14	−91.94	0.39
IR	2	188.14	1.33	0.51	0.13	−92.03	0.52
Population	2	188.76	1.94	0.38	0.10	−92.33	0.62
IR + sex	3	189.12	2.31	0.32	0.08	−91.47	0.70
Sex + population	3	189.81	3.00	0.22	0.06	−91.82	0.76
IR + population	3	190.16	3.35	0.19	0.05	−91.99	0.80
IR + sex + IR:sex	4	190.52	3.71	0.16	0.04	−91.11	0.84
Sex + population + sex:population	4	190.69	3.87	0.14	0.04	−91.19	0.88
IR + sex + population	4	191.05	4.24	0.12	0.03	−91.38	0.91
IR + population + IR:population	4	192.16	5.35	0.07	0.02	−91.93	0.93
IR + sex + population + sex:population	5	192.18	5.37	0.07	0.02	−90.86	0.95
Plasmodium							
Null	1	173.99	0.00	1.00	0.15	−85.98	0.15
IR	2	174.50	0.51	0.78	0.12	−85.21	0.27
IR + sex + IR:sex	4	174.57	0.57	0.75	0.11	−83.13	0.38
Sex	2	174.57	0.57	0.75	0.11	−85.24	0.49
IR + sex	3	174.71	0.72	0.70	0.10	−84.27	0.60
Population	2	175.71	1.72	0.42	0.06	−85.81	0.66
IR + sex + population + IR:sex	5	175.90	1.90	0.39	0.06	−82.72	0.72
Sex + population	3	176.05	2.05	0.36	0.05	−84.94	0.77
IR + sex + population	4	176.33	2.33	0.31	0.05	−84.01	0.82
IR + population	3	176.36	2.37	0.31	0.05	−85.09	0.86
IR + sex + population + sex:population + IR:sex	6	177.43	3.43	0.18	0.03	−82.40	0.89
Sex + population + sex:population	4	177.81	3.82	0.15	0.02	−84.76	0.91
IR + sex + population + IR:sex + IR:population	6	177.96	3.96	0.14	0.02	−82.66	0.93
IR + sex + population + sex:population	5	178.28	4.29	0.12	0.02	−83.92	0.95
Trypanosoma							
Population	2	104.25	0.00	1.00	0.21	−50.08	0.21
Sex + population + sex:population	4	104.90	0.66	0.72	0.15	−48.30	0.36
Null	1	105.30	1.06	0.59	0.12	−51.64	0.48
Sex + population	3	106.04	1.79	0.41	0.08	−49.93	0.56
IR + population	3	106.18	1.93	0.38	0.08	−50.00	0.64
IR + sex + population + sex:population	5	106.70	2.45	0.29	0.06	−48.12	0.70
Sex	2	106.74	2.49	0.29	0.06	−51.32	0.76
IR	2	107.10	2.85	0.24	0.05	−51.51	0.81
IR + sex + population	4	108.02	3.77	0.15	0.03	−49.86	0.84
IR + population + IR:population	4	108.29	4.04	0.13	0.03	−49.99	0.87
IR + sex + population + sex:population + IR:sex	6	108.36	4.11	0.13	0.03	−47.86	0.90
IR + sex	3	108.64	4.39	0.11	0.02	−51.23	0.92
IR + sex + population + IR:population + sex:population	6	108.84	4.59	0.10	0.02	−48.10	0.94
IR + sex + population + IR:sex	5	109.03	4.79	0.09	0.02	−49.29	0.96

95% confidence candidate sets are presented. The models within $\leq 2\Delta$ AICc (i.e., highly supported) are marked in bold

^aNumber of estimable parameters

^bAkaike information criterion corrected for small-sample size

^cDistance to the most parsimonious model in AICc units

^dAkaike weight (probability of a model)

Table 3 Estimates and odds ratios (OR) of the predictors included in the three candidate sets used to determine the best models explaining infection of Aquatic Warblers by any of the blood parasites pooled, only *Plasmodium* and only *Trypanosoma*, respectively

Model	Estimate (CI)	OR (CI)
Blood parasites pooled		
Intercept	−0.53 (−0.92, −0.13)	
IR	0.70 (−1.00, 2.40)	2.01 (0.37, 11.05)
Sex ^a	−0.21 (−0.62, 0.21)	0.81 (0.54, 1.23)
Population ^b	0.09 (−0.30, 0.48)	1.10 (0.74, 1.62)
IR:sex	−0.83 (−2.76, 1.10)	0.44 (0.06, 3.02)
IR:population	0.29 (−1.37, 1.95)	1.34 (0.25, 7.02)
Sex:population	0.23 (−0.20, 0.66)	1.26 (0.82, 1.94)
<i>Plasmodium</i>		
Intercept	−0.88 (−1.33, −0.44)	
IR	0.79 (−1.31, 2.89)	2.21 (0.27, 17.98)
Sex ^a	−0.28 (−0.74, 0.17)	0.75 (0.48, 1.19)
Population ^b	0.16 (−0.26, 0.57)	1.17 (0.77, 1.77)
IR:sex	−1.69 (−3.85, 0.47)	0.18 (0.02, 1.59)
IR:population	0.34 (−1.53, 2.21)	1.40 (0.22, 9.14)
Sex:population	0.17 (−0.31, 0.65)	1.19 (0.73, 1.92)
<i>Trypanosoma</i>		
Intercept	−2.24 (−3.01, −1.47)	
IR	−0.59 (−3.39, 2.20)	0.55 (0.03, 9.03)
Sex ^a	−0.07 (−0.87, 0.73)	0.93 (0.42, 2.08)
Population ^b	−0.44 (−1.20, 0.32)	0.65 (0.30, 1.38)
IR:sex	1.21 (−2.08, 4.51)	3.37 (0.12, 91.07)
IR:population	−0.07 (−3.25, 3.12)	0.93 (0.04, 22.54)
Sex:population	0.71 (−0.12, 1.53)	2.03 (0.89, 4.63)

Due to model selection uncertainty, the estimates were averaged over the models in the 95% confidence candidate sets

CI 95% confidence interval

^aMales relative to females

^bPolesie relative to Biebrza

Infection by *Trypanosoma* was best explained by the population model, while the only model containing IR within $\Delta AICc < 2$ ranked fifth, with a model probability of 0.08 (Table 2). The estimate of the IR effect was also not truly different from one (Table 3, Fig. 1c). The relative importance of IR, sex, and population in explaining *Trypanosoma* infection was 0.38, 0.51, and 0.73, respectively. The full model fitted the data well ($X^2 = 4.60$, $df = 8$, $p = 0.799$).

In all the analyses, the order and support for the models were nearly identical when they were run with standardized heterozygosity (SH) instead of IR (not shown).

Heterozygosity and blood parasites: single-locus effects

We found support that infection by blood parasites pooled was negatively affected by heterozygosity at locus AW-03.

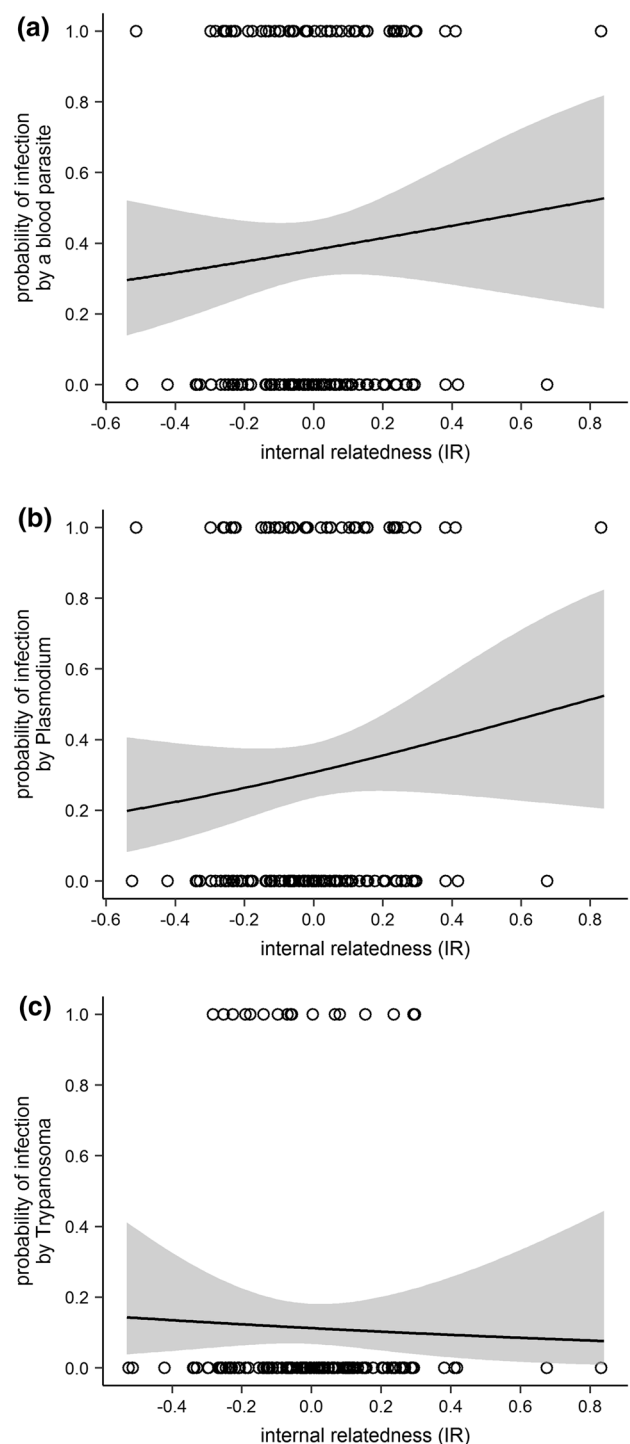


Fig. 1 Relationship between internal relatedness (IR, centred) and the probability of infection by **a** at least one of the blood parasites identified in the study (*Plasmodium*, *Trypanosoma*, or *Leucocytozoon*), **b** *Plasmodium*, and **c** *Trypanosoma*. The lines were fitted based on the estimates from the top models that included IR (Table 2). Bands show confidence intervals and the circles represent the distribution of samples (0—uninfected; 1—infected). None of the associations was clearly supported by the data (see Tables 2 and 3)

The AW-03 model was the only one within $\Delta\text{AICc} < 2$; it was 9.8 times more probable than the IR model and 5.6 times more probable than the constant infection model (Table 4). The AW-03 estimate was -0.96 (95% CI -1.81 to -0.12) and its OR was 0.38 (95% CI 0.16–0.89), indicating that individuals heterozygous at the AW-03 locus were 0.38 times less likely to carry blood parasite infection relative to homozygous individuals.

Infection by *Plasmodium* was best explained by the Ase19 and AW-03 models, which were the only ones within $\Delta\text{AICc} < 2$. The top model, containing the Ase19 locus, was 4.3 times more probable than the IR model and 3.3 times more probable than the null model (Table 4). The Ase19 estimate was -0.82 (95% CI -1.6 to -0.05), OR 0.44 (95% CI 0.20–0.95); the AW-03 estimate was -0.74 (95% CI -1.61 to 0.14), OR 0.48 (95% CI 0.20–1.15). Therefore, in individuals heterozygous at Ase19, the probability of infection with *Plasmodium* was 0.44 of that of homozygous individuals, while the effect of AW-03 was weakly supported, as the CIs of its OR spanned one.

There was considerable model selection uncertainty concerning *Trypanosoma* infection models, with none of the loci receiving clearly better support than the other loci or IR (Table 4). No estimates of the $\Delta\text{AICc} < 2$ models showed an effect on infection probability, as all of their 95% CIs spanned zero (not shown). In all the three analyses, the order and support for models was also very similar when SH was used instead of IR (not shown).

Discussion

Heterozygosity: genome-wide effects

We did not find strong evidence for an association between multi-locus heterozygosity and the incidence of infection by blood parasites in the Aquatic Warbler, a rare bird species that has gone through a dramatic decline in numbers and geographical range. Therefore, in our study, the relationship between MLH and infection status in this species cannot be convincingly explained by the general effects of heterozygosity. Still, heterozygosity appears to be a relatively important predictor of *Plasmodium* infection incidence in the Aquatic Warbler. Our results also indicate that heterozygosity has different importance as a predictor of infection by *Plasmodium* and by *Trypanosoma*, as it poorly explained infection by the latter.

Studies on birds that related infection by blood parasites to MLH have yielded mixed results. In Blue Tits (*Cyanistes caeruleus*), MLH at microsatellite markers showed a negative quadratic relationship with the probability of infection by *Plasmodium relictum* (Ferrer et al.

2014). In the Common Buzzard (*Buteo buteo*), MLH was correlated with infection by *Leucocytozoon buteonis* through interaction with vole abundance and age of individuals (Boerner et al. 2013). In American Crows (*Corvus brachyrhynchos*), lower heterozygosity was associated with infection of offspring, but not adults, by *Plasmodium* spp. (Townsend et al. 2018a). However, other avian studies failed to find any relationship (Ortego et al. 2007; Valender et al. 2012).

Our study was hampered by the lack of polymorphism of some of the microsatellite loci and presence of null alleles, which led to exclusion of 6 out of the 17 markers. The low final number of markers, together with their moderate polymorphism (Table 1), could explain why we did not find firm support for the effect of MLH on blood parasite infection status (Hansson and Westerberg 2002; Balloux et al. 2004; Slate et al. 2004; Ljungqvist et al. 2010; Forstmeier et al. 2012). On the other hand, it was also emphasized that finding an HFC is dependent on the variance in inbreeding rates (Hansson and Westerberg 2002; Csilléry et al. 2006; Ljungqvist et al. 2010; Taylor et al. 2010; Forstmeier et al. 2012). The isolation of breeding sites, the relatively high breeding philopatry, the skewed paternities and the recent steep decline of the Aquatic Warbler (Dyrce et al. 2002, 2005; Briedis and Keišs 2016; Bellebaum 2018; Flade et al. 2018) indicate that in this species, increased variation in inbreeding could be expected. Nevertheless, we found a low variance in inbreeding (g_2), which suggests low variation in relatedness in the studied areas.

It is also possible that MLH better explains quantitative infection rates, rather than infection probability, as the latter does not differentiate between acute and chronic infection. For example, in mountain White-crowned Sparrows (*Zonotrichia leucophrys oriantha*), the intensity of infection with hematozoan parasites was lower in more heterozygous individuals (MacDougall-Shackleton et al. 2005), and in Banded Mongooses (*Mungos mungo*), individuals with increased heterozygosity had lower intestinal parasite loads (Mitchell et al. 2017). Infection status might also be related to MLH only in a harsher environment (Boerner et al. 2013; Annavi et al. 2014). Finally, the genetic mechanism underlying resistance to blood parasites could be a trait determined by one or a few genes, making it less likely to observe correlation of genome-wide heterozygosity with blood parasite resistance (Szulkin et al. 2010; Bateson et al. 2016).

Heterozygosity: single-locus effects

We observed local effects of loci AW-03 and Ase19, which predicted a lower infection probability by the studied blood parasites taken together, and by *Plasmodium*, respectively,

Table 4 Model selection results for the relationship between probability of infection by blood parasites and single-locus heterozygosity

Model	K^a	AICc ^b	Δ AICc ^c	Evidence ratio	ω AICc ^d	Log-likelihood	Cumulative AICc
<i>Blood parasites pooled</i>							
AW-03	2	183.41	0.00	1.00	0.39	−89.66	0.39
AW-09	2	185.79	2.38	0.30	0.12	−90.85	0.51
AW-02	2	186.72	3.31	0.19	0.08	−91.32	0.59
Null	1	186.81	3.40	0.18	0.07	−92.39	0.66
Ase19	2	186.93	3.52	0.17	0.07	−91.42	0.73
AW-08	2	187.42	4.01	0.13	0.05	−91.67	0.78
AW-06	2	188.06	4.65	0.10	0.04	−91.99	0.82
IR	2	188.14	4.73	0.09	0.04	−92.03	0.86
AW-04	2	188.40	4.99	0.08	0.03	−92.16	0.89
Pocc8	2	188.61	5.20	0.07	0.03	−92.26	0.92
Fhu2	2	188.67	5.26	0.07	0.03	−92.29	0.95
AW-07	2	188.76	5.35	0.07	0.03	−92.33	0.97
AW-05	2	188.77	5.36	0.07	0.03	−92.34	1.00
<i>Plasmodium</i>							
Ase19	2	171.54	0.00	1.00	0.30	−83.72	0.30
AW-03	2	173.16	1.62	0.44	0.13	−84.53	0.43
Null	1	173.99	2.46	0.29	0.09	−85.98	0.52
AW-08	2	174.30	2.76	0.25	0.08	−85.11	0.60
IR	2	174.50	2.96	0.23	0.07	−85.21	0.67
AW-06	2	174.91	3.37	0.19	0.06	−85.41	0.72
AW-02	2	174.95	3.42	0.18	0.05	−85.43	0.78
Pocc8	2	175.03	3.49	0.17	0.05	−85.47	0.83
AW-09	2	175.67	4.13	0.13	0.04	−85.79	0.87
AW-05	2	175.76	4.22	0.12	0.04	−85.84	0.90
Fhu2	2	175.90	4.36	0.11	0.03	−85.90	0.94
AW-07	2	176.02	4.48	0.11	0.03	−85.97	0.97
AW-04	2	176.05	4.52	0.10	0.03	−85.98	1.00
<i>Trypanosoma</i>							
Null	1	105.30	0.00	1.00	0.15	−51.64	0.15
AW-09	2	106.00	0.70	0.70	0.11	−50.96	0.26
AW-03	2	106.23	0.93	0.63	0.10	−51.07	0.36
Fhu2	2	106.65	1.34	0.51	0.08	−51.28	0.44
AW-02	2	106.78	1.48	0.48	0.07	−51.35	0.51
AW-04	2	106.88	1.58	0.45	0.07	−51.40	0.58
AW-05	2	106.94	1.63	0.44	0.07	−51.42	0.65
IR	2	107.10	1.80	0.41	0.06	−51.51	0.71
Pocc8	2	107.20	1.89	0.39	0.06	−51.55	0.77
AW-07	2	107.23	1.92	0.38	0.06	−51.57	0.83
Ase19	2	107.25	1.94	0.38	0.06	−51.58	0.89
AW-08	2	107.32	2.01	0.37	0.06	−51.61	0.94
AW-06	2	107.34	2.04	0.36	0.06	−51.63	1.00

IR Internal relatedness^aNumber of estimable parameters^bAkaike information criterion corrected for small-sample size^cDistance to the most parsimonious model in AICc units^dAkaike weight (probability of a model)

when in heterozygous state. This indicates that AW-03 and Ase19 are in linkage disequilibrium with unidentified fitness loci, which confer a heterozygote advantage in the Aquatic Warbler.

Linkage disequilibrium between selected and apparently neutral loci can arise due to selection, population sub-structuring, and genetic bottleneck. However, unless recombination is low or suppressed, LD is disrupted with time (Hartl and Clark 2007). The severe decline experienced by the Aquatic Warbler during the last 100–150 years (Briedis and Keišs 2016; Flade et al. 2018) could have increased LD in its genome. Yet, our analyses of the same samples do not support a historical or recent genetic bottleneck in the study populations and no population sub-structuring was found between them (Kubacka unpubl.). Consequently, it is plausible that the association between heterozygosity of Ase19 and AW-03 and infection status in the Aquatic Warbler stems from their linkage with functional loci, such as those related to parasite resistance or infection clearance. Earlier research provides support for the association between Ase19 and immunity. In nestlings of the Bluethroat (*Luscinia svecica*), heterozygosity at this marker positively correlated with wing-web swelling following injection of phytohaemagglutinin (PHA) (Fossøy et al. 2009). In birds, response to PHA is associated with both innate and adaptive (T-cells) components of the immune system (Palacios et al. 2009; Vinkler et al. 2010; Salaberria et al. 2013). It is also positively related to the probability of recovery from infection with *Haemoproteus sp.* (Gonzalez et al. 1999).

In general, our results add to the increasing evidence that single-locus effects could underlie HFCs more than it was acknowledged earlier. Single-locus effects have been reported in previous HFC studies on wild-ranging animals (Hansson et al. 2004; Lieutenant-Gosselin and Bernatchez 2006; Ortego et al. 2007; Kaeuffer et al. 2008; Fossøy et al. 2009; Vangestel et al. 2011; García-Navas et al. 2014; Minias et al. 2015; Judson et al. 2018). However, only a few HFC studies related to disease documented local effects (Acevedo-Whitehouse et al. 2006, 2009; Amos and Acevedo-Whitehouse 2009) and, to our knowledge, our study is the first to show them in a disease-related study on a bird.

The local effects of Ase19 and AW-03 could help indicate genomic regions that are under parasite-mediated selection, as pointed out by Acevedo-Whitehouse et al. (2009). In addition, as they suggest a heterozygote advantage at functional genes, they also imply that factors affecting genetic diversity in the Aquatic Warbler, such as the constant loss of habitats and the resulting genetic drift, could impact blood parasite resistance in this species. Finally, these local effects can be applied in conservation management of the species to identify individuals or populations resistant to blood

parasites, for instance in translocation projects (Grueber et al. 2008).

Conclusion

While we found that internal relatedness at microsatellite loci appears to be an informative predictor of *Plasmodium* infection in the Aquatic Warbler, our results do not provide a solid support for a negative inbreeding effect on blood parasitism in this species. However, they demonstrate a case for single-locus effects on disease probability in birds, which, to our knowledge, has not been reported before. For the Aquatic Warbler, the observed local effects could have meaningful conservation implications. In general, they add to the accumulating evidence that local effects could underpin apparent genome-wide HFCs.

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Data availability The full data set generated during this study is available from the corresponding author on reasonable request. The input files and the R code used in the analysis have been published in the Mendley Data repository (<https://doi.org/10.17632/rcb8czrpfj.1>).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed involving animals were in accordance with the ethical standards of the institution at which the studies were conducted. The protocol and procedure of blood-sampling were ethically reviewed and approved of by the 1st Ethical Committee in Lublin, Poland (decision no. 17/2014 of 25 April 2014). The birds were caught and ringed under permissions from the Polish Ministry of the Environment (decision no. DLP-III-4102343125042/14/md and ringing permit no. 144/2014), the Biebrza National Park, the Poleski National Park, and the Regional Directorate for Environmental Protection in Lublin (permits no. WPN.6401.104.2014.MPR and WPN.6205.1.23.2014.MO), as required by the Polish Nature Protection Act.

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